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Far-red fluorescence: A direct spectroscopic marker for LHCII oligomer formation in non-photochemical quenching

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Abstract Time-resolved fluorescence on oligomers of the main light-harvesting complex from higher plants indicate that in vitro oligomerization leads to the formation of a weakly coupled inter-trimer chlorophyll–chlorophyll (Chl) exciton state which converts in tens of ps into a state which is spectrally broad and has a strongly far-red enhanced fluorescence spectrum. Both its lifetime and spectrum show striking similarity with a 400 ps fluorescence component appearing in intact leaves of *Arabidopsis* when non-photochemical quenching (NPQ) is induced. The fluorescence components with high far-red/red ratio are thus a characteristic marker for NPQ conditions in vivo. The far-red emitting state is shown to be an emissive Chl–Chl charge transfer state which plays a crucial part in the quenching.
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Keywords: LHCII; Aggregation; Chlorophyll fluorescence; Non-photochemical quenching; Violaxanthin; Zeaxanthin; Arabidopsis mutant

1. Introduction

The most abundant protein in the thylakoid membrane, the light-harvesting complex of photosystem II (LHCII), plays a crucial role in controlling the structure of the membrane and in the adaptation to light changes. On the one hand light-energy is absorbed by LHCII and transferred to the reaction center of photosystem II (PSII), on the other hand Lhcb proteins are involved in the non-photochemical quenching (NPQ) of excess light-energy in order to protect PSII from photodamage. A large part of NPQ is the so-called energy-dependent component (q_E) that is rapidly reversible (see e.g. [1–4] for recent reviews).

The energy-dependent NPQ is correlated with the lowering of the thylakoid lumen pH [1,5], the conversion of violaxanthin (Vx) to zeaxanthin (Zx) via the xanthophyll cycle [6,7] and the correlated action of the PsbS protein [8]. Following earlier studies of in vitro LHCII oligomers [9–11] (for a review see [12]) comparison studies of the excited state quenching in a LHCII crystal [13] and of in vitro LHCII oligomers [14] have led to the suggestion that oligomerization of LHCII and conformational changes [15] are responsible for the major part of q_E. The proposed mechanism involves an aggregation-induced conformational change of the LHCII protein which then allows energy transfer quenching by a lutein [14]. This interpretation is discussed rather controversially in the literature however. An alternative suggestion for both the localization as well as the mechanism of q_E quenching has been put forward [16–18]. According to this suggestion q_E involves Zx – located in the minor antenna complexes of PSII – acting as a direct quencher due to electron transfer resulting in the formation of a $Zx^{+\cdot}$ radical cation and a $Chl^{-\cdot}$ radical anion.

Elucidation of the detailed quenching mechanisms would be largely facilitated by the presence of spectroscopic markers which can be observed both in vivo under NPQ conditions and in vitro experiments. One such marker is the change in a neoxanthin Raman band occurring upon NPQ induction in vivo [14] which is however difficult to detect. We have thus started to search for other possible spectroscopic markers which might provide further hints on the photochemical mechanism(s) involved in NPQ. We report here a study on the time-resolved fluorescence of various in vitro LHCII oligomers (aggregates) and comparison of their spectroscopic properties to a characteristic fluorescence component that appears in vivo in intact *Arabidopsis* leaves under high light irradiation (NPQ) conditions. In both cases a characteristic far-red fluorescence component of similar lifetime is observed. We suggest that this far-red fluorescence is indicative of the formation of a Chl–Chl charge transfer (CT) state in the LHCII oligomers and that the strikingly similar far-red fluorescence under NPQ conditions provides a spectroscopic marker for a similar state in vivo in a NPQ mechanism.

2. Materials and methods

2.1. Isolation procedures

LHCII lamellar aggregates were isolated from spinach following the procedure described earlier [19,20]. LHCII trimers were isolated from the *npq1* and *npq2* mutants from *Arabidopsis thaliana* as described [21]. The *npq1* mutant cannot convert Vx to Zx due to a lack of the enzyme

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Abbreviations: Chl, chlorophyll; cmc, critical micelle concentration; CT, charge transfer; DAS, decay associated spectra; DM, dodecyl maltoside; LHCI, light-harvesting complex of photosystem I; LHCII, major light-harvesting complex of photosystem II; NPQ, non-photochemical quenching; Nx, neoxanthin; PSI, photosystem I; PSII, photosystem II; SAS, species-associated spectra; Vx, violaxanthin; Zx, zeaxanthin

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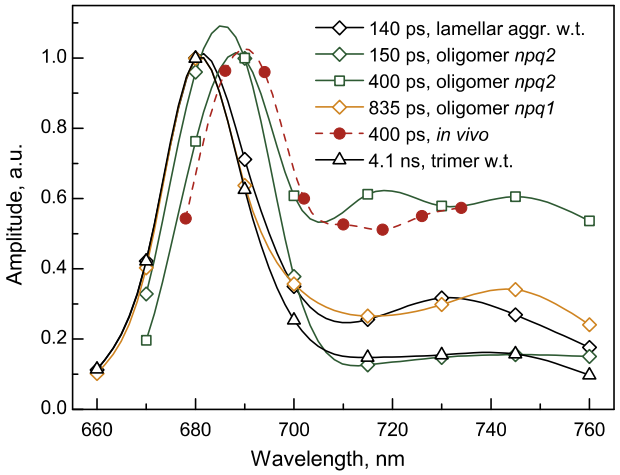

Fig. 1. Comparison of the DAS of selected lifetime components of various in vitro oligomers and of lamellar aggregates. The spectrum of the 400 ps in vivo lifetime component appearing under NPQ conditions in intact Arabidopsis leaves is also shown (red circles). Note that the latter spectrum has been corrected for self-absorption in the optically dense plant leaf using a correction function that has been obtained from a comparison of the total fluorescence spectra from the leaf and that of isolated thylakoids from the same plant. All spectra are normalized to the maximum.

Vx-deepoxidase, while the *npq2* mutant produces Zx already in the dark due to a lack of Zx-epoxidase. The far-red fluorescence lifetime component appearing after high light irradiation in vivo (cf. Fig. 1) has been measured in intact *Arabidopsis* w.t. leaves after irradiation with 600 μE/m^2/s photon flux density for 45 min. Low-temperature (77 K) fluorescence was measured in a cryostat with 60% glycerol added.

2.2. Time-resolved fluorescence

Time-resolved fluorescence measurements were performed as described in [22]. The fluorescence decays were recorded using a single-photon-counting apparatus with a resolution of 1–2 ps. The excitation wavelength was 645 nm and fluorescence was detected at different wavelengths between 660 and 760 nm. The fluorescence decays were analyzed by global lifetime analysis and by target analysis [23]. The LHCII trimers were measured in a buffer containing 10 mM HEPES pH 7.6, 0.06% β-dodecyl maltoside (DM). For the oligomers the buffer contained 30 mM $MgCl_2$ and 100 mM KCl instead of β-DM. All measurements were carried out at room temperature in a sealed measuring cuvette with an oxygen scavenging system added containing 1.36 mg/ml glucose, 65 μg/ml glucose-oxidase and 65 μg/ml catalase.

3. Results

3.1. Global lifetime analysis of the fluorescence kinetics

The fluorescence kinetics of the various trimeric and oligomeric LHCII complexes were measured in the range from ~660 to ~760 nm. The full decay associated spectra (DAS) of the fluorescence lifetime measurements of the trimeric complexes are given in the Supplementary Information (Fig. S1). The kinetics of the trimers was characterized by either four or five exponentials. The predominant amplitude component has a long lifetime of 4.1 and 3.8 ns (spinach w.t. and *npq1*-LHCII from Arabidopsis, respectively) whereas the *npq2*-LHCII has a slightly shortened long lifetime of 3.5 ns. The shortest-lived exponential of 7–8 ps arises from an excited state equilibration.

Three different types of aggregated LHCII samples were compared: oligomers obtained from isolated trimers of the *npq1* and *npq2 Arabidopsis* mutants and lamellar macroaggregates isolated from spinach leaves. The latter type of preparation contains in addition minor antenna complexes and a relatively high lipid amount [19]. The lamellar aggregates, organized in multilayer membrane-like sheets have been shown to mimic features of the native thylakoid membranes [20,24]. The full set of fluorescence DAS for the oligomers is given in Fig. S2 (Supplementary Information). The kinetics of these LHCII oligomers required 4–5 exponentials for a good fit. In the spectrum of the lamellar aggregates from spinach (Fig. S2A) the main lifetime component is 650 ps whereas the longest component is 1.1 ns with relative amplitude of 26% (at the spectral maximum near 685 nm). The 140 ps component shows an enhanced far-red/red amplitude ratio (as compared to the ratio in LHC II trimers). The DAS of the LHCII-*npq2* complex (Fig. S2B) revealed a different profile. Instead of one major lifetime component, two fast lifetimes of 400 and 150 ps have similarly high amplitudes around 685 nm. Only one of them, the 400 ps component, shows a high far-red/red amplitude ratio. Similar to the spinach lamellar aggregates, the longest lifetime was found to be 1.2 ns, but with a much smaller amplitude. The average lifetime is the shortest-lived among all the oligomers, i.e. 284 ps. Fig. S2C shows the DAS of the LHCII-*npq1* oligomers. The main lifetime component is 835 ps with a substantially higher far-red/red amplitude ratio than the other components. Fig. 1 shows the DAS of the main lifetime components from the different oligomers and trimers, selected to compare their different far-red to red ratios (for the full set of DAS see Figs. S1 and S2). It illustrates that some lifetime components in the oligomers have a pronouncedly enhanced far-red (710–740 nm) to red (680–690 nm) amplitude ratio, both in relation to the other components in the oligomers, but in particular in comparison to the DAS of the long-lived (ns) trimer fluorescence. The most strongly enhanced far-red fluorescence is observed in LHCII-*npq2* oligomers in their 400 ps component. Fig. 1 also shows for comparison the DAS of an approx. 400 ps lifetime component that appears in vivo in intact *Arabidopsis* leaves upon irradiation with high light (NPQ conditions). Such a red-shifted and strongly far-red-enhanced (far-red to red amplitude ratio) fluorescence lifetime component is absent in dark-adapted leaves. Overall the oligomers are strongly quenched as compared to the trimers, as can be seen in their much shorter average fluorescence lifetimes (cf. Figs. S1 and S2).

3.2. Target analysis of the kinetics

A close inspection of the kinetic data from the oligomers reveals that in particular in oligomers of LHCII-*npq1* and LHCII-*npq2* the far-red enhanced fluorescence components are formed within a few tens of ps from some of the lifetime component(s) that have spectra which are more similar to those of the trimeric species. We have thus performed a kinetic model analysis (so-called target analysis, [23]) where two emitting species are connected by a reversible process, which could either be an energy transfer process or some other process leading to a new emitting state (cf. Fig. 2). This target analysis allows us to determine the rate constants of the forward and backward reactions, the decay rate of the second state, and the corresponding species-associated spectra

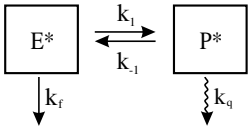

Fig. 2. Two-state model of the fluorescence kinetics of LHCII oligomers. The rate constants k_1, k_{-1}, and k_q were determined by global target analysis (cf. Table 1). The rate constant k_F has been varied parametrically over a range of values from which 0.3–0.4 ns^{-1} gave the best fitting results. The states E^* and P^* are proposed to be a Chl–Chl exciton state formed between LHCII trimers and a Chl–Chl charge transfer (CT) state with a dominant excited state character, respectively.

(SAS). Indeed the fluorescence kinetics of the oligomers – except for the small amounts of very long-lived components – could be fitted very well with such a model containing two connected emitting species. If the second species was assumed to be non-emitting in the analysis, no reasonable fit to the data could be obtained. The resulting spectra are shown in Fig. 3 and the rate constants in Table 1. We have ignored in this analysis some small amplitude component(s) with lifetimes of a few ps. The two connected emitting states show quite different SAS. The initially excited state has a spectrum that peaks slightly to the red (by 2–5 nm) and is slightly broader than the DAS of the long-lived component in LHCII trimers. This points to the formation of an initial emitting state in the oligomers that is different from the trimers. The far-red fluorescence intensity of this initial component (E^*) – as compared to the red fluorescence amplitude – and its spectral shape are

Table 1
Rate constants k_i [ns^{-1}] (cf. model in Fig. 2) and lifetimes τ_i [ps] resulting from the analysis of a homogeneous oligomer model

	k_1	k_{-1}	k_q	τ_1[a]	τ_2	τ_3	τ_4
LHCII-*npq1* oligomers	47	56	3.0	**10**	**675**	220	1200
LHCII-*npq2* oligomers	16	18	4.8	**27**	**445**	180	800

[a]The two lifetimes indicated in bold in each case are those that result from the two-state model shown in Fig. 2. The other two lifetimes (indicating components with small amplitudes) are required as additional independent components for a good fit.

quite similar to that of the long-lived (ns) component in LHCII trimers. In contrast the fluorescence spectrum of the product state (P^* in Fig. 2) is further red-shifted in its peak to about 685 nm. The most striking feature is however a very broad and pronounced far-red tail (710–760 nm) that is responsible for the far-red enhanced steady state fluorescence of the oligomers (Fig. S3). The SAS of the target model are quite similar for the corresponding components of the oligomers of the *npq1* and *npq2* trimers. However, the rate constants of equilibration between the two emitting states as well as the deactivation rate of the P^* state differ for the two oligomers.

4. Discussion

In the present work we analyze the combined effect of the LHCII aggregation and the Zx presence on the fluorescence lifetime of LHCII. In contrast to previous fluorescence kinetic

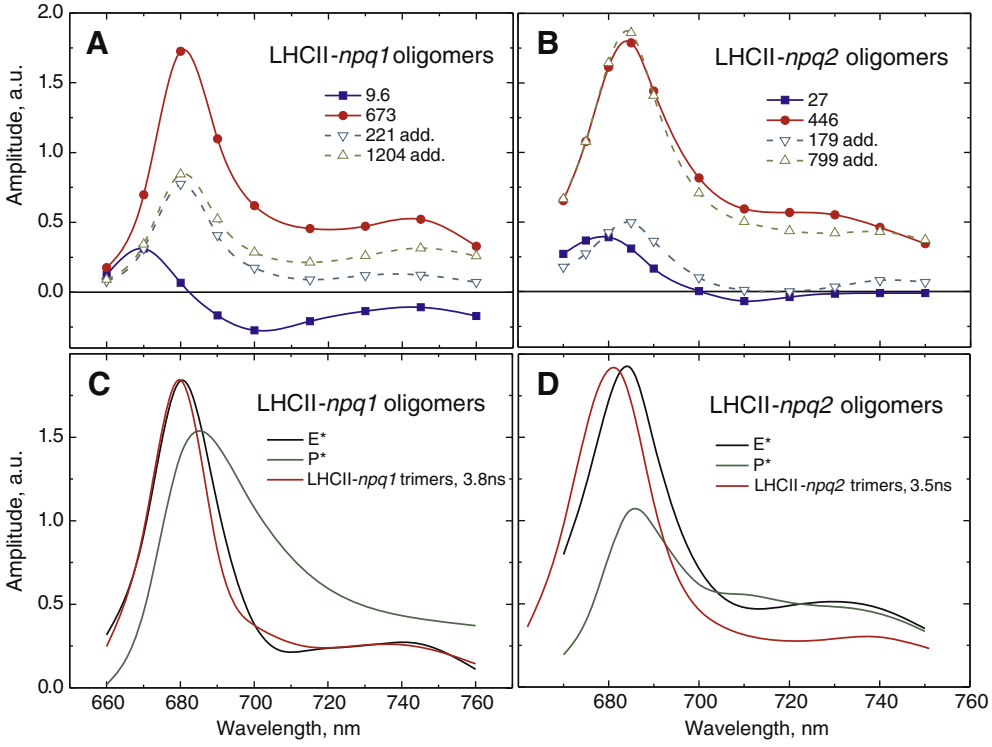

Fig. 3. DAS (A and B) and SAS (C and D), resulting from a homogeneous CT model for the oligomers (cf. Fig. 2) of *npq1* (A and C) and *npq2* (C and D) LHCII. The DAS of the long (ns) lifetime component of the corresponding LHCII trimers is also shown for comparison in red. In some cases a better fit can be obtained by a heterogeneous model having two exciton and charge-transfer states. The results of such fit are shown for *npq2* in Figure S5 in the Supplementary Information.

studies on LHCII complexes, the V1 site, which is supposed to be involved in the NPQ mechanism, is fully occupied. Thus the complexes accommodate Vx and lutein in LHCII-w.t. and LHCII-*npq1*, and Zx and lutein in LHCII-*npq2*, allowing us to directly address the question of the role of Zx bound to its physiologically relevant site (Note that in *npq2* a very small amount of Zx is found additionally in the L1 site [25]).

At the level of trimeric LHCII the binding of Zx leads to a slight shortening of the long (nanosecond) Chl lifetime. Because in this complex V1 is fully occupied with Zx and a small amount of Zx also binds to L1, this represents the maximal possible effect of Zx in the V1 site. This direct quenching effect of Zx is however much too small to account for the large NPQ observed in vivo. We do not exclude that it may enhance slightly the major part of quenching which must be due to a different effect however.

The data on LHCII in vitro oligomers confirm that the Chl fluorescence lifetime is drastically quenched after aggregation of the LHCII trimers. It has been reported in several different studies [11,26–28] that the longest lifetime of 3–4 ns for the trimeric LHCII is reduced to about 1 ns within the oligomers. This is in agreement with our results on the oligomers from *npq1* (which contain no Zx as did all previous LHCII aggregates) and from lamellar aggregates. The LHCII-*npq2* oligomers containing Zx were more quenched than previously reported aggregates (average lifetime of 280 ps). Furthermore, in our data the major amplitude component(s) in these oligomers have generally shorter lifetimes than in previous studies. However, it should be noted that the mean lifetimes varied slightly between preparations, as well as measurement conditions, e.g. sample concentration (data not shown). Also, a small amount of unquenched trimers could largely affect the mean lifetime. Such variations are well-known for aggregated LHC II preparations. More important than the overall lifetimes/yields in the oligomers are the spectral features of the lifetime components.

As a new feature that has not been reported so far, some short lifetime components in oligomers show a strongly enhanced far-red/red amplitude ratio (Fig. 3 and Fig. S2) as compared to LHCII trimers. This far-red enhancement occurs both in *npq1* and *npq2* mutants, but is most pronounced in *npq2*. A similar far-red fluorescence has also been observed in LHCII crystals [13]. It was interpreted as indicating a change in Chl configuration within a trimer in the crystal leading to different emitting species. LHCII has a large fraction of Chls that are located at or close to the surface of the protein [29,30]. While we cannot exclude the possibility of a change in the Chl–Chl interaction within a trimer upon oligomerization, we believe that it is much more likely that oligomerization leads to new Chl–Chl interactions among close-lying Chl molecules in different trimers. This is for example clearly the case in the crystals where, depending upon the crystal form, Chl pairs are created with shortest π–π (edge-to-edge) distances of about 6 Å [29] and 15 Å [30]. While in vitro oligomers are clearly expected to be much less ordered and are likely somewhat heterogeneous in their relative arrangement, it is reasonable to assume that Chl–Chl pairs of similar or even shorter distances as found in crystals can be formed in such oligomers (Note that the decay lifetimes have relatively narrow distributions (Fig. S4) signifying a small heterogeneity of the oligomers).

These new inter-trimer Chl–Chl pairs form weakly coupled exciton states, as is indicated by the slight bathochromic shift and a slight broadening of the short-lived DAS in the oligomers relative to the spectra of the trimers. Such spectral features are typical of Chl–Chl exciton states. However, the very broad and far-red enhanced fluorescence component can not be explained by a Chl–Chl exciton state alone. Strong excitonic coupling in aggregates has been excluded based on the fact that apart from a small (1–2 nm) red shift the absorption spectrum remains the same as compared to isolated trimers [31]. Even if we assumed formation of strongly-coupled excitonic states, this could not explain the very broad shape of the spectra in the far-red range since excitonic interactions generally lead to spectral narrowing rather than broadening [32]. Rather the broad spectral shape and the far-red enhancement are characteristic of the formation of a charge transfer (CT) state that still maintains a pronounced excited state character, i.e. the charge is – on average – only partially translocated from the donor to the acceptor Chl. In contrast, formation of a complete radical pair state can be confidently ruled out since Chl radicals are non-fluorescent quenchers and would thus not be detected as emitting species in the time-resolved spectra. However, our analysis shows clearly an emissive state, albeit with lower oscillator strength (this can be estimated from the area under the SAS) than a typical Chl excited state, pointing to its mixed exciton/CT state character.

Mixing of CT states with Chl–Chl exciton states leads to a drastic change in their spectroscopic properties including a large Stokes shift, very strong broadening, and strong vibrational tails [33–35]. All these effects are due to the very strong electron–phonon-coupling of these states. Such effects have been observed earlier and were shown to originate from bacteriochlorophyll and Chl excited states having strong CT character in bacterial antenna systems, PSI cores from cyanobacteria [36–38] and in LHCI [34,39,40]. In fact the so-called "red chlorophylls" in the PSI antenna that were proven to have strong CT character, share striking common spectroscopic features with the far-red emission spectra of the LHCII oligomers. The extremely low efficiency of hole-burning of these Chls [34] has been related to the large electron-phonon coupling [33]. Interestingly, in a hole-burning study of LHCII oligomers, Pieper et al. [41] have suggested that the low hole-burning efficiency beyond 683 nm has the same origin, i.e. strong electron-phonon coupling due to CT state character.

A comparison of the 77 K fluorescence spectra of LHCII trimers and oligomers shows two new bands relative to the 682 nm fluorescence of LHCII trimers (Fig. 4): (i) the exciton emission band located at 683–684 nm and (ii) the very broad far-red fluorescence peaking at 701 nm. According to the Gaussian fits of the spectra (shown as dotted curves in Fig. 4), the bandwidth of the first component is similar to that in isolated trimers – 200 cm^{-1} – whereas the second component, which reflects the mixed exciton/CT state emission, has a bandwidth of about 600 cm^{-1} and a long tail extending to the far-red. Detailed comparison reveals that this bandwidth is even larger than the width of the "red LHCI chlorophylls" of 400 cm^{-1} [40]. Although part of the extreme broadening may be due to inhomogeneous broadening, i.e. a heterogeneity in the spectra, this could clearly not explain the full extent of the broadening. Thus this extremely broad low temperature spectrum provides the strongest and clearest evidence that this state has CT character. The broad fluorescence component distinctly appears in LHCII with or without Zx – both at room

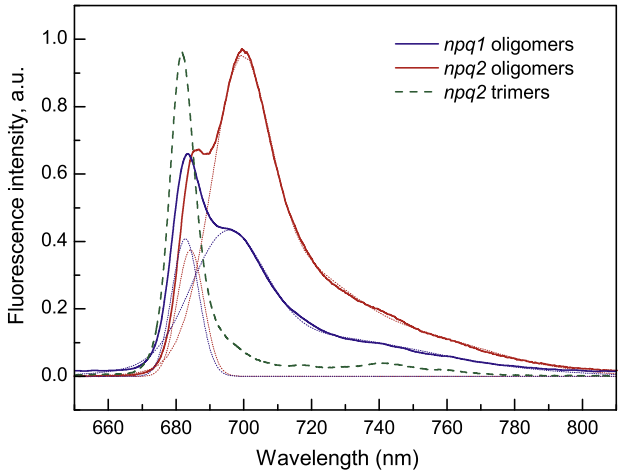

Fig. 4. Fluorescence emission spectra of *npq1*- and *npq2*-LHCII oligomers and trimeric *npq2*-LHCII, registered at 77 K. Note the formation of two new emission bands upon oligomerization vs. the single 681 nm emission peak of the trimer. The dotted curves represent Gaussian fit of the short and long wavelength bands in the oligomeric spectra. Peak positions are 682 nm and 696 nm for *npq1* and 684 nm and 700 nm for *npq2* (red). Bandwidths (FWHM) are 200 cm^{-1} and 610 cm^{-1} for the short and long wavelength bands, respectively.

temperature and at low temperature – but it is most pronounced in Zx-containing samples (*npq2*), possibly because Zx favours particular LHCII interactions.

The emissive character of the CT state implies a relatively moderate coupling of the Chl pairs (probably in the order of a few tens of cm^{-1}) and also a not very pronounced asymmetry between the two Chl components. This CT state is formed within 10 ps (*npq1* oligomers) or 27 ps (*npq2* oligomers) from the initial Chl–Chl exciton state at room temperature and the two states are in equilibrium with each other, implying that the energy of the CT state is in the vicinity of the exciton state energy. The CT state itself is strongly quenched with decay rates between 3 and 5 ns^{-1} (cf. Table 1). Fluorescence data alone cannot provide the final fate of the CT state. The quenching could be caused either by a pronounced coupling of the CT state to the ground state, a situation that would be quite typical for CT states, or it could be quenched by energy transfer to another low-lying state.

The far-red enhanced fluorescence of the LHCII oligomers in vitro derives from the emitting CT state which is the lowest energy emitting state in the system. It is striking that a nearly identical emission spectrum and lifetime (ca. 400 ps) are observed in vivo under NPQ conditions (cf. Fig. 1) as is found in the *npq2*-LHCII oligomers. We note here that Gilmore et al. also resolved a ca. 400 ps component appearing under NPQ conditions in spinach thylakoids but did not resolve it spectrally [42]. We observed this 400 ps fluorescence component in intact *Arabidopsis* leaves only under NPQ conditions while it is missing in dark-adapted leaves. Its lifetime and spectral shape differ pronouncedly from the properties of any other known higher plant antenna complex [43]. Thus it can be clearly correlated with the formation of a new state characteristic for NPQ. We thus propose that the far-red-enhanced 400 ps fluorescence in vivo under NPQ conditions reflects the formation of a LHCII oligomer giving rise to an emitting CT state. This CT state plays a crucial role in the NPQ process either as a direct quencher or as a quenching intermediate. Our data suggest that Zx has a supporting effect on the formation of the quenched oligomeric state but is not strictly required. It has been shown earlier that Zx can stimulate the formation of LHCII oligomers whereas Vx functions as an inhibitor for oligomerization [44].

From the similarity of the in vitro CT state properties to the properties of the far-red fluorescence component observed in intact leaves in NPQ we suggest that it provides a direct spectroscopic marker for the induction of the LHCII oligomer quenching state in vivo. The arrangement and interactions between LHCII trimers in NPQ in vivo and in the in vitro oligomers do not need to be – and likely are not – identical. Yet the resulting spectral effects which are due to the formation of the CT state caused by the trimer–trimer interaction, and the quenching intermediates appear to be very similar. This can be understood based on the not very critical orientation dependence of the CT state. The CT state formation and properties will be controlled much more by the polarity of the environment rather than the orientation dependence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.09.044.

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